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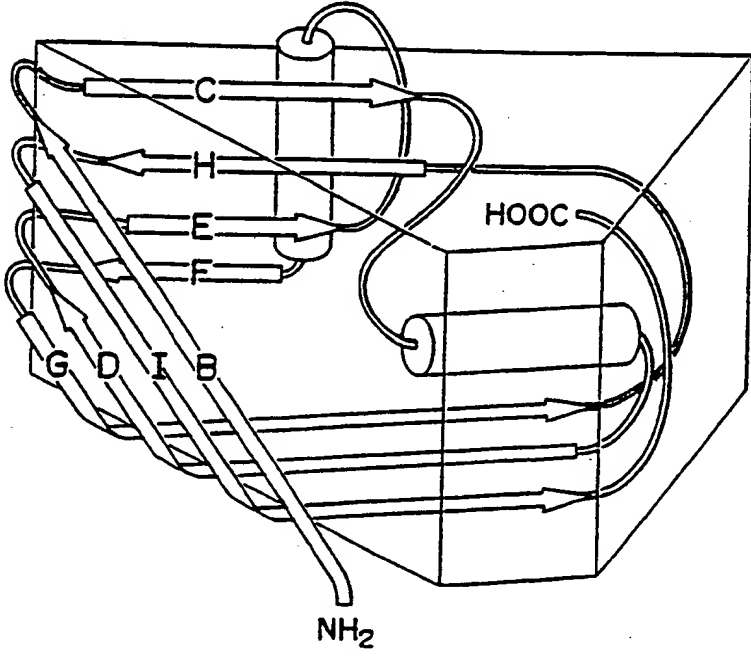
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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| <p>(21) International Application Number: PCT/GB92/00589<br/>(22) International Filing Date: 2 April 1992 (02.04.92)<br/>(30) Priority data: 9108386.5 19 April 1991 (19.04.91) GB<br/>(71) Applicants (for all designated States except US): AGRICULTURAL GENETICS COMPANY LIMITED [GB/GB]; 154 Science Park, Milton Road, Cambridge CB4 4GG (GB). PURDUE RESEARCH FOUNDATION [US/US]; Office of Technology Transfer, 328 Engineering Administration Building, West Lafayette, IN 47907 (US).<br/>(72) Inventors; and<br/>(75) Inventors/Applicants (for US only) : LOMONOSSOFF, George, Peter [GB/GB]; Yohn Innes Centre for Plant Science Research, Colney Lane, Norwich NR4 7UH (GB). JOHNSON, John, Emil [US/US]; Department of Biological Sciences, Purdue University, West Lafayette, IN 47907 (US).</p> |           | <p>(74) Agent: ELKINGTON AND FIFE; Prospect House, 8 Pembroke Road, Sevenoaks, Kent TN13 1XR (GB).<br/>(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, RU, SD, SE, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent), US.<br/><br/>Published<br/>With international search report.</p> |
| <p>(54) Title: MODIFIED PLANT VIRUSES AS VECTORS</p>  <p>(57) Abstract</p> <p>The invention relates to assembled particles of a plant virus containing a predetermined foreign peptide as part of the coat protein of the virus, and a method for their production. The foreign peptide is preferably a biologically functional peptide, the biological application of which requires or is enhanced by presentation of the peptide in association with a larger molecule or particle.</p>  |           |   |

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### MODIFIED PLANT VIRUSES AS VECTORS

This invention relates to the use of viruses as carriers (vectors) for the production or presentation of foreign peptides. More particularly, the invention relates to the genetic manipulation of viral nucleic acid by incorporation of foreign nucleic acid sequences which are expressed as peptides in the virus particle (virion). In this specification the term "foreign", as applied to a peptide or to the nucleic acid encoding therefor, signifies peptide or nucleic acid sequences which are not native to the plant virus used as a vector. Such sequences can be alternatively described as exogenous or heterologous sequences. The term "peptide" includes small peptides and polypeptides.

The use of viruses as carriers of foreign peptides has been extensively explored in the field of composite virus vaccines. Such vaccines are based on chimeric viruses which are hybrids of different animal virus components. Usually the major component of such hybrids is derived from a virus which is or has been rendered harmless and the minor component is a selected antigenic component of a pathogenic virus. For example, a pox virus such as vaccinia or an attenuated poliovirus may be used as a vector for immunogenic components of other animal viruses including human viruses.

However, the above technique has several disadvantages. Such vaccines are produced from viruses grown in cell culture systems which are expensive to design and run. The composite virus approach involves genetic manipulation of live, animal-infecting viruses, with the risk that mutations may give rise to novel forms of the virus with altered infectivity, antigenicity and/or pathogenicity. The animal virus used as the vector is often a virus to which the animal may already have been exposed, and the animal may already be producing antibodies to the vector. The vector may therefore be destroyed by the immune system before the incorporated antigenic site of the second virus induces an immune response.

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The present invention avoids the above-mentioned disadvantages by the use of a radically different type of virus component in the design of chimeric viruses expressing foreign sequences. Moreover, although the invention has particular relevance to the solution of problems encountered in the production of virus vaccines, it is much wider both in concept and field of application as indicated hereinafter.

The present invention utilises plant viruses as vector systems for the expression of foreign nucleotide sequences ie nucleotide sequences (RNA or DNA) which are not present in plant viruses, as found in Nature, and which in consequence code for peptides not normally found in any naturally occurring plant virus.

The present invention comprises assembled particles of a plant virus containing a foreign peptide. The plant viruses of the present invention are therefore modified forms of the native viruses and for convenience will be referred to as modified viruses.

The foreign peptides which may be incorporated into plant viruses according to this invention may be of highly diverse types and are subject only to the limitation that the nature and size of the foreign peptide and the site at which it is placed in or on the virus particle do not interfere with the capacity of the modified virus to assemble when cultured *in vitro* or *in vivo*. In broad concept, modified viruses may be formed from any biologically useful peptides (usually polypeptides) the function of which requires a particular conformation for its activity. This may be achieved by association of the peptide with a larger molecule eg to improve its stability or mode of presentation in a particular biological system. Examples of such peptides are peptide hormones; enzymes; growth factors; antigens of protozoal, viral, bacterial, or fungal origin; antibodies including anti-idiotypic antibodies; immunoregulators and cytokines eg interferons and interleukins; receptors; adhesins; and parts or precursors of any of the foregoing types of peptide. The peptide preferably contains more than 5 amino acids.

Among the broad range of bioactive peptide sequences bound to plant virus vectors in accordance with the present invention special importance attaches to the antigenic peptides which are the basis of vaccines, particularly animal (including human) virus vaccines. For vaccine applications the invention provides an especially attractive epitope presentation system. When used for such applications the antigenic peptide component will be sited appropriately on the virus particle so as to be easily recognised by the immune system, for example by location on an exposed part of the coat protein of the virus. As applied to the latter, therefore, the present invention comprises assembled particles of a modified plant virus containing an antigen derived from a pathogen eg an animal virus, incorporated in an exposed surface of the coat protein of the plant virus. The invention also comprises the use of such assembled modified plant virus particles as the immunogenic component of a vaccine.

In US patent 4,407,956 and European applications 067,553 194,809 221,044 and PCT applications WO 87/06261 and WO/90/00611 it has previously been proposed to use the DNA and RNA of certain plant viruses as vectors for the introduction of exogenous material into plants either to confer new properties on the plant itself or to enable certain valuable compounds (eg plant metabolites) to be recovered. In such prior proposals interest focuses entirely on the modified DNA or RNA of the plant virus and its use in the phenotypic transformation of plants. Most of these prior specifications are of a theoretical and speculative nature and none discloses the isolation of whole particles (virions) of a plant virus having a modified coat protein or the use of such particles for the preparation of an animal virus vaccine, or for any other purpose. In Febs Letters (1990) 269, 73-76 Takamatsu et al describe attempts to express a foreign peptide of 5 amino acids fused to the carboxy-terminus of the coat protein of tobacco mosaic virus. However, no modified plant viral particles were produced in the experiments described.

To produce modified plant virus particles in accordance with this invention the plant viral nucleic acid is modified by introducing a nucleotide sequence coding for the foreign peptide eg an animal virus antigen at that part of the plant viral

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genome which codes for an exposed portion of the coat protein, infecting plants or plant cells with the modified viral nucleic acid, and harvesting assembled particles of the modified virus. This procedure is best carried out by direct manipulation of the DNA of the virus in the case of DNA viruses or by manipulation of a cDNA corresponding to the RNA of an RNA virus. In the case of an RNA virus, an RNA transcript of the modified DNA is usually prepared for inoculation of plant cells or preferably whole plants so as to achieve a multiplication stage prior to the harvesting of assembled particles of the modified virus. In the case of a DNA virus, the DNA itself is introduced into the plant. In this way, the foreign peptide is initially expressed as part of the capsid protein and is thereby produced as part of the whole virus particle. The peptide may thus be produced as a conjugate molecule intended for use as such. Alternatively, the genetic modification of the virus may be designed in order to permit release of the desired peptide by the application of appropriate agents which will effect cleavage from the virus particle.

In order to produce modified virus on a commercial scale, it is not necessary to prepare infective inoculant (DNA or RNA transcript) for each batch of virus production. Instead, an initial inoculant may be used to infect plants and the resulting modified virus may be passaged in the plants to produce whole virus or viral RNA as inoculant for subsequent batches.

For the purposes of the present invention a particularly valuable group of viruses for use as vectors are those in which the nucleic acid coding for the capsid is a separate moiety from that which codes for other functional molecules and whose coat proteins have a  $\beta$ -barrel structure. An advantage of the use of viruses which have this structure is that the loops between the individual strands of  $\beta$ -sheet provide convenient sites for the insertion of foreign peptides. Modification of one or more loops is a preferred strategy for the expression of foreign peptides in accordance with the present invention. This group includes the comoviruses such as cowpea mosaic virus and bean pod mottle virus, and the nepoviruses such as tomato ringspot virus and strawberry latent ringspot virus. An advantage of the comoviruses is that their capsid contains sixty copies

each of 3 different  $\beta$ -barrels which can be individually manipulated. Other virus groups with similar 3-dimensional structures but a single type of  $\beta$ -barrel include the tombusviruses and the sobemoviruses. Other groups of plant and animal viruses which share structural similarities but whose coat proteins do not have a  $\beta$ -barrel structure may also be modified in accordance with this invention, for example the plant and animal rhabdoviruses.

The foreign RNA or DNA may be inserted into the plant virus genome in a variety of configurations. For example, it may be inserted as an addition to the existing nucleic acid or as a substitution for part of the existing sequence, the choice being determined largely by the structure of the capsid protein and the ease with which additions or replacements can be made without interference with the capacity of the genetically-modified virus to assemble in plants. Determination of the permissible and most appropriate size of addition or deletion for the purposes of this invention may be achieved in each particular case by experiment in the light of the present disclosure. The use of addition inserts appears to offer more flexibility than replacement inserts in some instances.

In accordance with this invention, multiplication of modified virus and production of significant yields thereof in plant hosts is an important part of the novel strategy of the invention to produce antigens for vaccines and other types of peptide in an advantageous manner. As indicated above, the inserted heterologous nucleotide sequence may include those coding for amino acids which are readily cleaved so that, after the multiplication stage, the desired material may be separated from the virus particles. As an alternative to total cleavage of the peptide, it may be possible and desirable in some cases to release the peptide in a form in which it remains intact within a major part of the capsid but separated from the viral nucleic acid.

Included among the many epitopes which can be expressed on the surface of the CPMV capsids are those from picornaviruses such as foot-and-mouth disease virus (FMDV), poliovirus, human rhinovirus (HRV) and hepatitis A virus (HAV), epitopes associated with either gp41 or gp120 of human immunodeficiency virus



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(HIV) and the epitope derived from the major coat protein of human papillomavirus (HPV).

As applied to the preparation of vaccines, the present invention has many advantages over conventional vaccines, recombinant vaccines based on animal viruses, and peptide vaccines, for example:

1. Lower production costs, as very high yields of pure virus are obtainable from infected plants, and no tissue culture production step is necessary.
2. Improved safety, as plant viruses are incapable of infecting and replicating in animals, and thus will not be able to mutate into virulent forms, as may be the case with conventional and recombinant animal virus vaccines.
3. Some plant viruses, particularly comoviruses, are exceptionally stable, and purified preparations can be dried and stored for many years at room temperature without losing infectivity. This property will allow the development of slow-release vaccines, reducing the number of injections required to maintain immunity.
4. Animals are unlikely to have been exposed to plant viruses, and therefore will not already have antibodies to the vector, thus increasing the effectiveness of the composite vaccine.
5. The plant viruses, being smaller than most of the animal viruses which have been previously used as vectors eg vaccinia, allow the introduction of chimeric genes by *in vitro* manipulation as contrasted with homologous recombination *in vivo* (transfection).

To demonstrate this system, the plant virus cowpea mosaic comovirus (CPMV) was chosen. The three-dimensional structure of the CPMV has been solved at atomic resolution which has enabled identification of sites suitable for modification without disruption of the particle structure.

To demonstrate the wide applicability of this invention, antigenic sites of three different animal viruses were used. Two were viruses belonging to the picornavirus group of animal viruses - foot and mouth disease virus (FMDV) and human rhinovirus (HRV). There are several important pathogens in this group, particularly FMDV, poliomyelitis (polio) and hepatitis A.

The third virus selected was human immune deficiency virus (HIV) which bears no similarity to any known plant virus, and for which no successful vaccines are currently available.

The invention will now be further described with reference to the following accompanying drawings:

**Figure 1** depicts a comparison of the structures of simple T=3 virus, picornavirus and comovirus capsids. In each case, one trapezoid represents one  $\beta$ -barrel. Thus the large (L or VP37) capsid protein of comoviruses consists of two covalently linked  $\beta$ -barrels which are equivalent to the C and B-type subunits of a T=3 virus or VP2 and VP3 of picornaviruses. The small (S or VP23) capsid protein of comoviruses contains a single  $\beta$ -barrel which corresponds to the A-type subunits of a T=3 virus or VP1 of a picornavirus.

**Figure 2** depicts the secondary structure and connectivity of a canonical  $\beta$ -barrel. The individual strands of  $\beta$  sheet are labelled B through G and the amino- ( $\text{NH}_2$ ) and carboxy- ( $\text{COOH}$ ) termini of the protein are indicated.

**Figure 3** depicts the plasmids (A) pPMM2902 and (B) pBT7-123. The stippled regions represent the CPMV-specific regions of the plasmids with the coding regions being indicated by the wider portions on which the various virus-encoded proteins are marked. Relevant restriction enzyme sites are indicated. Details of the construction of the plasmids are given in Holness *et al* (1989) and Dessens and Lomonossoff (1991).

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**Figure 4** depicts the region of CPMV M RNA which encodes the amino-terminal 40 amino acids of VP23. The numbers below the nucleotide sequence refer to the M RNA sequence and the position of the unique Nhe1 site is indicated. The amino acids involved in forming the  $\beta$ B and  $\beta$ C strands of VP23 are indicated above the amino acid sequence of the protein which is shown using the standard one-letter code.

**Figure 5** depicts (A) the nucleotide sequence of the oligonucleotides used in the construction of pFMDV together with the amino acid sequence encoded by the top (positive) strand and (B) the structure of VP23 after insertion of the FMDV-specific oligonucleotides. The arrowed region indicates the extent of the inserted FMDV epitope. The Nhe1 site not restored during the cloning is indicated by xNhe1. The diagnostic Bgl11 site present in the inserted sequence is also indicated.

**Figure 6** depicts the construction of plasmid pFMDV. The representation of the various CPMV-specific regions is as in Figure 3. The FMDV-specific region which is inserted into VP23 is shown as the black segment in the CPMV-specific coding region.

**Figure 7** depicts plasmid pMT7-601. The representation of the various CPMV-specific regions is as in Figure 3. Relevant restriction sites are indicated.

**Figure 8** depicts the construction of a "substitution" vector by site-directed mutagenesis. The asterisk indicates the T residue that is changed to a C by site-directed mutagenesis, thereby creating a novel Aat11 site.

**Figure 9** depicts (A) the nucleotide sequence of the oligonucleotides used in the construction of pMT7-HIV together with the amino acid sequence encoded by the top (positive) strand and (B) the structure of VP23 after insertion of the HIV-specific oligonucleotides. The arrowed region indicates the extent of the inserted HIV epitope. The diagnostic Pvu1 site present in the inserted sequence is also indicated.

Figure 10 depicts (A) the nucleotide sequence of the oligonucleotides used in the construction of pMT7-HRV together with the amino acid sequence encoded by the top (positive) strand and (B) the structure of VP23 after insertion of the HRV-specific oligonucleotides. The arrowed region indicates the extent of the inserted HRV epitope. The diagnostic ClaI site present in the inserted sequence is also indicated.

Figure 11 depicts the construction of plasmids pMT7-HIV and pMT7-HRV. The representation of the various CPMV-specific regions is as in Figure 3. The HIV- and HRV-specific regions inserted into VP23 are shown as the black segment in the CPMV-specific coding region.

Figure 12 depicts a "Western blot" of protein extracts of the leaves of five cowpea plants (lanes A to E) inoculated with a mixture of pMT7-FMDV-1 and PBT7-123 transcripts. The blot was probed with serum specific for the FMDV epitope. Lanes "mock" and "wt" contain extracts of leaves which were either mock-inoculated or inoculated with wild-type CPMV RNA, respectively. The lane marked "virus" contains purified wild-type CPMV. The sizes of marker proteins are shown on the right-hand side of the blot.

### Comoviruses

Comoviruses are a group of at least fourteen plant viruses which predominantly infect legumes. Their genomes consist of two molecules of single-stranded, positive-sense RNA of different sizes which are separately encapsidated in isometric particles of approximately 28nm diameter. The two types of nucleoprotein particles are termed middle (M) and bottom (B) component as a consequence of their behaviour in caesium chloride density gradients, the RNAs within the particles being known as M and B RNA, respectively. Both types of particle have an identical protein composition, consisting of 60 copies each of a large (VP37) and a small (VP23) coat protein. In addition to the nucleoprotein

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particles, comovirus preparations contain a variable amount of empty (protein-only) capsids which are known as top (T) component.

In the case of the type member of the comovirus group, cowpea mosaic virus (CPMV), it is known that both M and B RNA are polyadenylated and have a small protein (VPg) covalently linked to their 5' terminus. More limited studies on other comoviruses suggest that these features are shared by the RNAs of all members of the group. Both RNAs from CPMV have been sequenced and shown to consist of 3481 (M) and 5889 (B) nucleotides, excluding the poly (A) tails (van Wezenbeek et al. 1983; Lomonossoff and Shanks, 1983). Both RNAs contain a single, long open reading frame, expression of the viral gene products occurring through the synthesis and subsequent cleavage of large precursor polypeptides. Though both RNAs are required for infection of whole plants, the larger B RNA is capable of independent replication in protoplasts, though no virus particles are produced in this case (Goldbach et al., 1980). This observation, coupled with earlier genetic studies, established that the coat proteins are encoded by M RNA.

A 3.5Å electron density map of CPMV shows that there is a clear relationship between CPMV and the T=3 plant viruses such as the tombusviruses, in particular tomato bushy stunt (TBSV) and the sobemoviruses, in particular southern bean mosaic (SBMV). The capsids of these latter viruses are composed of 180 identical coat protein subunits, each consisting of a single  $\beta$ -barrel domain. These can occupy three different positions, A, B and C, within the virions (Figure 1). The two coat proteins of CPMV were shown to consist of three distinct  $\beta$ -barrel domains, two being derived from VP37 and one from VP23. Thus, in common with the T=3 viruses, each CPMV particle is made up of 180  $\beta$ -barrel structures. The single domain from VP23 occupies a position analogous to that of the A type subunits of TBSV and SBMV, whereas, the N- and C- terminal domains of VP37 occupy the positions of the C and B type subunits respectively (Figure 1).

X-ray diffraction analysis of crystals of CPMV and another member of the group, bean pod mottle virus (BPMV) shows that the 3-D structures of BPMV and CPMV are very similar and are typical of the comovirus group in general.

In the structures of CPMV and BPMV, each  $\beta$ -barrel consists principally of 8 strands of antiparallel  $\beta$ -sheet connected by loops of varying length. The connectivity and nomenclature of the strands is given in Figure 2. The flat  $\beta$ -sheets are named the B,C,D,E,F,G,H and I sheets, and the connecting loops are referred to as the  $\beta$ B- $\beta$ C,  $\beta$ D- $\beta$ E,  $\beta$ F- $\beta$ G and  $\beta$ H- $\beta$ I loops.

The comoviruses are also structurally related to the animal picornaviruses. The capsids of picornaviruses consist of 60 copies of each of three different coat proteins VP1, VP2 and VP3 each one consisting of a single  $\beta$ -barrel domain. As in the case of comoviruses, these coat proteins are released by cleavage of a precursor polyprotein and are synthesised in the order VP2 - VP3 - VP1. Comparison of the 3-dimensional structure of CPMV with that of picornaviruses has shown that the N- and C- terminal domains of VP37 are equivalent to VP2 and VP3 respectively and that VP23 are equivalent to VP1 (Figure 1). The equivalence between structural position and gene order suggests that VP37 corresponds to an uncleaved form of the two picornavirus capsid proteins, VP2 and VP3.

One of the principal differences between the comoviruses and picornaviruses is that the protein subunits of comoviruses lack the large insertions between the strands of the  $\beta$ -barrels found in picornaviruses though the fundamental architecture of the particles is very similar. The four loops ( $\beta$ B- $\beta$ C,  $\beta$ D- $\beta$ E,  $\beta$ F- $\beta$ G and  $\beta$ H- $\beta$ I - see Figure 2) between the  $\beta$ -sheets are not critical for maintaining the structural integrity of the virions but, in accordance with this invention, are used as sites of expression of foreign peptide sequences, such as antigenic sites from animal viruses.

### Modification of CPMV virus

In order to make insertions into the coat protein of CPMV, it is necessary to have a means of manipulating the genome of the virus. A full-length cDNA clone of CPMV M RNA (pPMM2902) in the transcription vector pPMI was available (see Figure 3A) (Ahlgquist and Janda, 1984, Holness et al. (1989) and Holness (1989). We have shown that transcripts from pPMM2902 can multiply when electroporated in cowpea mesophyll protoplasts in the presence of highly purified virion B RNA, therefore allowing modifications to be made to the viral coat proteins without affecting the multiplication and assembly of the virus.

In view of the possible danger that B RNA purified from virions to provide the proteins required for viral replication with pPMM2902 might be cross-contaminated with wild-type M RNA, we have constructed a full-length cDNA clone of B RNA, pBT7-123 (see Figure 3B). The full-length copy of B RNA is immediately downstream of a modified T7 promoter. Following linearisation with the restriction enzyme Mlu1, transcripts identical in size to natural B RNA can be synthesised by T7 RNA polymerase. A mixture of transcripts from pPMM2902 and pBT7-123 gives rise to a full virus infection when electroporated into cowpea protoplasts, and therefore replaces the use of natural B RNA.

We have selected the  $\beta$ B- $\beta$ C loop in VP23 for the insertion of foreign peptide. This loop is clearly exposed on the surface of the viral particle and computer modelling has shown that even large loops inserted at this site are unlikely to interfere with the interaction between adjacent subunits responsible for capsid structure and stability. This loop has a unique Nhe1 site at position 2708 of the M RNA-specific sequence where foreign sequences may be inserted (see Figure 4).

The principle antigenic sites of the picornavirus foot and mouth disease (FMDV) and human rhinovirus (HRV), and the lentiretrovirus human immune deficiency virus (HIV) were used to illustrate the use of this invention in the production of vaccines to animal viruses.

**Design and construction of pFMDV, a full length cDNA clone of CPMV M RNA containing a DNA insert coding for a segment of FMDV loop protein**

To insert the "FMDV loop" into the  $\beta$ B- $\beta$ C loop of VP23 of CPMV, two complementary oligonucleotides, both 81 residues long, were chemically synthesised. Their sequences are given in Figure 5A. The positive sense oligonucleotide contains the sequence encoding amino acid residues 136-160 from VP1 of FMDV serotype O, strain BFS 1860. The nucleotide sequence of oligonucleotides was designed to take account of the codon usage preference found in CPMV and includes a Bgl11 site in the middle of the sequence to facilitate screening. When annealed, the oligonucleotides give a double-stranded DNA sequence with Nhe1-compatible ends. Thus the oligonucleotides can be inserted into the unique Nhe1 site of pPMM2902. The effect of such an insertion on the sequence of the VP23 is shown in Figure 5B. To facilitate the insertion of the FMDV loop, the FMDV-specific oligonucleotides were initially ligated into an M13 sub-clone of pPMM2902 which contained the sequence encoding VP23. This was done to enable clones harbouring the FMDV-specific sequence to readily be identified by sequence analysis. All the standard DNA manipulations were carried out according to Maniatis *et al* (1982). Details of the construction of pFMDV are given below and are shown diagrammatically in Figure 6.

STEP 1. The plasmid pPMM2902 was digested with the restriction enzyme Sst1 which cuts twice within the CPMV M RNA-specific sequence at positions 2296 and 3423 but does not cut within the sequence of the plasmid pPM1. Following agarose gel electrophoresis, and both the large (6.0kb) the small (1.1kb) fragment were purified by electroelution from the gel. The 1.1kb Sst1 fragment was ligated into the Sst1-cut, phosphatase-treated replicative form DNA from the bacteriophage M13mp18. The ligation mixture was used to transform *E.coli* strain JM101 using the calcium chloride procedure. Plaques containing the 1.1kb Sst1 fragment from M RNA were identified by the Lac complementation assay and DNA sequence analysis and one, M13-JR1 was selected for further work.



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STEP 2. The double-stranded replicative form DNA of M13-JR1 was isolated from infected E. coli strain JM101 cells by the method of Birnboim and Doly (1979). The purified DNA was linearised by digestion with the restriction enzyme Nhe1 and the linearised plasmid treated with calf intestinal phosphatase. The two oligonucleotides with Nhe1-compatible termini encoding amino acid residues 136 to 160 of VP1 from FMDV were phosphorylated with ATP using polynucleotide kinase and annealed to each other by boiling and slow cooling. The annealed oligonucleotides were ligated into Nhe-1-digested M13-JR1, the ligation mixture used to transform E. coli strain JM101 and the transformation mixture plated out on a lawn of JM101. A large number of plaques were found on the plates, 20 of which were selected for sequence analysis. Bacteriophage were propagated in JM101 and the single-stranded DNA isolated exactly as described by Sanger et al (1980). The nucleotide sequence of the region of the bacteriophage DNA around the Nhe1 site was determined by the dideoxy method as modified by Biggin et al (1983), using an 18mer, 5' AGT-TAC-TGC-TGT-AAC-GTC-3', complementary to nucleotides 2735-2752 of the M RNA sequence, as primer. Of the plaques analysed, one, designated M13-usha1, had a single copy of the desired sequence in the correct orientation.

STEP 3. M13-usha1 was propagated in E.coli strain JM101 and the replicative form DNA was isolated from the infected cells by the method of Birnboim and Doly (1979). The DNA was digested with Sst1 and the 1.1kb fragment purified by agarose gel electrophoresis. This fragment was ligated to the large (6.0kb) Sst1 fragment from pPMM2902 (see above) which had been treated with calf intestinal phosphatase. The ligation mixture was used to transform E.coli strain JM83 using the calcium chloride method. The transformation mixture was plated out on L-agar plates containing 100 µg/ml carbenicillin and the plates incubated overnight at 37°C. 12 carbenicillin-resistant colonies were selected for further study. The colonies were grown up as 1ml cultures in L-broth, plasmid "minipreps" prepared and analysed by restriction enzyme digestion. From the patterns obtained by digestion with the enzymes Sst1, Bgl11 and EcoRV, it was possible to deduce that 4 colonies consisted of full-length clones of CPMV containing the sequence of the FMDV-specific oligonucleotides in the correct

orientation. One of these, pFMDV, was subsequently propagated on a large scale and the plasmid DNA was isolated by the method of Birnboim and Doly (1979) and further purified by centrifugation using caesium chloride/ethidium bromide gradients (Maniatis et al (1982).

### **Properties of pFMDV transcripts**

1. Purified pFMDV DNA was linearised by digestion with EcoR1 and transcribed using E.coli RNA polymerase exactly as described for pPMM2902 by Holness et al (1989). Electrophoresis of the products of formaldehyde-containing agarose gels (Lehrach et al., 1977) revealed the presence of transcripts which co-migrated with authentic viral M RNA.
2. Following treatment with DNase1 and lithium chloride precipitation to remove the template DNA, the transcripts were translated in vitro in the message-dependent rabbit reticulocyte system (Pelham and Jackson 1976) in the presence of <sup>35</sup>S-methionine. The products were examined by electrophoresis on polyacrylamide gels containing SDS (Laemmli, 1970) and visualised by autoradiography of the dried-down gel. The autoradiographs revealed the presence of two proteins of 105 and 95kDa which co-migrated with the translation products of natural M RNA.
3. The ability of transcripts of pFMDV to be replicated in plant cells was examined as follows: Cowpea mesophyll protoplasts were prepared as described by de Varennes et al (1985). Transcripts from pFMDV were mixed with transcripts from pBT7-123 (the plasmid containing a full-length copy B RNA) and electroporated into the protoplasts as described by Holness et al (1989). As control, a sample of the same preparation of protoplasts were electroporated with a mixture of transcripts from pBT7-123 and pPMM2902. 72 hours post-electroporation the protoplasts were harvested and the nucleic acids were extracted as described by de Varennes et al (1985). Samples of the RNA were electrophoresed on formaldehyde-containing agarose gels (Lehrach et al., 1977) and the nucleic acids blotted on to Hybond N membranes (Amersham

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International). The nucleic acids were cross-linked to the membranes by irradiation with u.v. light. The membranes were probed for M RNA sequences using a HindIII fragment from pPMM2902 corresponding to nucleotides 482-2211 of the M RNA which had been labelled with  $^{32}\text{P}$  as described by Feinberg and Vogelstein (1983). Samples from protoplasts electroporated with pBT7-123 and either pPMM2902 or pFMDV-1 revealed the presence of M RNA-specific sequences confirming that the presence of the sequence encoding the FMDV loop did not prevent the transcripts from replicating. To confirm that the progeny of pFMDV replication retained the sequence encoding the FMDV loop, replicate membranes were probed with the positive-sense FMDV oligonucleotide which had been "oligo-labelled" (Feinberg and Vogelstein, 1983) to give a (+) sense-specific probe. The sample from protoplasts electroporated with a mixture of pBT7-123 and pFMDV transcripts gave a clear signal at the expected position for M RNA, a signal which was absent from the pPMM2902 control.

4. To establish that protein subunits containing the FMDV loop assemble into virions, extracts from infected protoplasts were examined for the presence of virus particles by immunosorbent electron microscopy. Samples of protoplasts electroporated with a mixture of transcripts from pBT7-123 and pFMDV were lysed by repeated passage through a 23 gauge needle. The extracts were centrifuged in an Eppendorf microfuge and supernatant retained for examination. 10 microlitre samples of the supernatants were incubated with gold electron microscope (em) grids which had been coated with anti-CPMV antiserum. After washing and staining with uranyl acetate, the grids were examined using a JEOL 1200 electron microscope. Particles of diameter 28nm could be seen which had the characteristic appearance of CPMV virions. This demonstrates that the presence of the FMDV loop in VP23 does not prevent virus assembly.

The foregoing description establishes that plant viruses modified in accordance with this invention can multiply and assemble into virus particles when electroporated into plant protoplasts. To produce modified plant viruses on a large scale it is necessary to prepare a construct which can be inoculated directly

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onto whole plants, and which will replicate and assemble into virus particles as in the above described protoplast system. We have therefore modified pPMM2902 in such a way that the resulting transcripts incorporate a "cap" structure at their 5' ends, and RNA synthesis is driven by a more efficient promoter. The steps in the modification of pPMM2902 to produce pMT7-601 (Figure 7) are described in detail below.

### Development of a system capable of infecting whole cowpea plants

#### Construction of pMT7-601

1. 1st strand cDNA to purified CPMV M RNA was synthesised exactly as described by Lomonossoff et al (1982), using pdT<sub>12-18</sub> as a primer. 2nd strand synthesis was primed using the following oligonucleotide:

|   |             |                |
|---|-------------|----------------|
| Pst1  | T7 Promoter | ↓ 5' end M RNA |
| 5' CCCTGCAGTAATACGACTCACTATAGTATTAATAATCTTAATAG |             |                |

Conditions for synthesis were as described in Lomonossoff et al (1982) and Shanks et al (1986).

2. The double-stranded cDNA was digested with the restriction enzymes Pst1 and BamH1 (which cleaves the M RNA sequence at position 1504) and the 1.5kb Pst1/BamH1 fragment ligated into Pst1/BamH1 digested M13mp18. The ligation mix was used to transform E.coli strain JM101. Recombinant phage harbouring inserts were identified by the lac complementation assay and checked for the presence of the correct insert by "T-track" analysis (Sanger et al., 1980) as modified by Biggin et al (1983). One clone, M13-MT7-6, was selected for further analysis and the sequence of the 5' terminal 200 nucleotides of M RNA specific sequence was determined as described by Biggin et al (1983) and shown to be identical to the equivalent sequence in pPMM2902.

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3. The double-stranded, replicative DNA was isolated from E.coli JM101 cells infected with M13-MT7-6 by the method of Birnboim and Doly (1979). The double stranded DNA was digested with Pst1 and Bgl11 (which cuts the M RNA sequence at position 189) and the 200bp fragment released was purified by electrophoresis on and electro-elution from an agarose gel (Maniatis et al (1982).

4. The plasmid pPMM2902 (Holness et al 1989) was digested with Pst1 and Bgl11 to produce two DNA fragments of 1.1 and 6.0kb. The smaller (1.1kb) fragment contains the sequence of the E.coli promoter linked to the first 189 nucleotides of the sequence of CPMV M RNA while the larger (6.0kb) fragment the rest of the sequence of M RNA linked to pUC9. The digest was treated with calf intestinal phosphatase, the two fragments separated by agarose gel electrophoresis and the 6.0kb fragment recovered by electro-elution.

5. The 200bp Pst1/Bgl11 from M13-MT7-6 and the 6.0kb fragment from pPMM2902 were ligated together (Maniatis et al, 1982) and the mixture used to transform E.coli strain JM83. A number of carbenicillin-resistant colonies were identified and one, pMT7-601, was shown to have the desired structure. Large-scale quantities of plasmid pMT7-601 were therefore prepared as described for pFMDV.

6. After linearisation with EcoR1, plasmid pMT7-601 could be transcribed using T7 RNA polymerase to give RNA which was identical in size to natural virion M RNA when analysed on formaldehyde-containing agarose gels (Lehrach et al. 1977). The yield of transcript was approximately 1 µg of full-length M transcript per µg of linearised template DNA.

7. When a mixture of T7 transcripts from pMT7-601 and pBT7-123 was electroporated into cowpea mesophyll protoplasts, Northern blot analysis of progeny RNA revealed that transcripts from pMT7-601 are biologically active. The methods used for protoplast isolation and nucleic acid analysis were identical to those used to analyse the biological properties of pFMDV.

**Infectivity of a mixture of capped pBT7-123 and pMT7-601 transcripts on cowpea plants**

Samples of pBT7-123 and pMT7-601 were linearised with MluI and EcoRI respectively. Portions of the linearised templates were transcribed using T7 RNA polymerase in the presence of GpppG essentially as described by Ziegler-Graaf et al (1988). The transcription reactions contained 0.1mg/ml linearised DNA template, 40mM Tris-HCl pH 8.0, 25mM NaCl, 8mM MgCl<sub>2</sub>, 2mM spermidine hydrochloride, 0.5mM each of UTP, ATP and CTP, 0.025mM GTP, 0.5mM GpppG, 0.05mg/ml BSA, 10mM DTT, 200 units/ml RNAGuard and transcription was initiated by the addition of T7 RNA polymerase to a final concentration of 1400 units/ml. Incubation was at 37°C for 2 hours. At 30, 60 and 90 minutes portions (5µl per 1 ml transcription reaction) of a 5mM solution of GTP were added. Following transcription, EDTA was added to 15mM final concentration and the integrity of the transcripts was checked by electrophoresis on formaldehyde-containing agarose gels. The transcription mixtures were extracted with 2 volumes of phenol/chloroform (1.1 v/v) and the nucleic acids precipitated twice with ethanol. The nucleic acids were harvested by centrifugation, washed with ethanol and dried under vacuum. The nucleic acids were dissolved in 50mM Tris-phosphate, pH 8.0 for inoculation on to plants.

The primary leaves of 10 day-old cowpea (Vigna unguiculata var. California blackeye) were dusted with carborundum and a 1:1 (w/w) mixture of transcripts derived from pMT7-601 and pBT7-123 were applied to the leaves with gently rubbing. A variety of transcript concentrations were used but in all cases the final inoculum volume was 50µl. The results obtained showed that when a total of 5µg of each transcript was applied per primary leaf, 100% of plants inoculated routinely developed symptoms characteristic of a CPMV infection. The presence of CPMV-specific sequences in both the inoculated and upper leaves of such plants was confirmed by "Dot blot" analysis. Samples of the inoculated and trifoliate leaves were taken using a number 10 cork borer and macerated and extracted with 0.4 mls of 10mM sodium phosphate. The samples were centrifuged and 5µl of the supernatant was applied to nitrocellulose filters pre-

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wetted with 20XSSC. The nucleic acid were cross-linked to the membranes by irradiation with u.v. light and probed for M RNA-specific sequences using a  $^{32}\text{P}$  "oligo-labelled" (Feinberg and Vogelstein, 1983) probe consisting of nucleotides 482-2211 of the M RNA sequence. The conditions for hybridisation and washing of the filters were as described by Maniatis *et al* (1982). After drying, the filters were autoradiographed. A strong hybridisation signal indicated the presence of CPMV-specific sequences.

#### **Construction of pMT7-FMDV-I, pMT7-HIV and pMT7-HRV**

To construct pMT7-FMDV-I, pMT7-601 and pFMDV were both digested with restriction enzyme Sst1, the digest from pMT7-601 being subsequently treated with calf intestinal phosphatase. Sst1 cuts each plasmid twice at positions 2296 and 3423 of the M RNA-specific region to release a 1.1kb fragment. As discussed previously this Sst1 fragment contains the region of VP23 encompassing the  $\beta\text{B}$ - $\beta\text{C}$  loop where the FMDV loop insertion has been made. Following electrophoresis on an agarose gel, the 1.1kb fragment from pFMDV and the 5.1kb fragment, encompassing the vector sequence and all the rest of the M RNA specific sequence, from pMT7-601 were recovered by electro-elution. The two Sst1 fragments were ligated together and the mixture transformed in *E. coli* strain JM83. A number of carbenicillin-resistant colonies were picked, "minipreps" made and the plasmid DNA examined by restriction enzyme digests to identify recombinants containing the FMDV loop. One such clone was identified, designated pMT7-FMDV-I and grown up on a large scale. All the DNA manipulations were as described for the construction of pFMDV and pMT7-601.

Both pFMDV and its derivative pMT7-FMDV-I have a straightforward insertion into the  $\beta\text{B}$ - $\beta\text{C}$  loop of VP23. To limit the increase in size of the loop upon insertion of a foreign sequence, a replacement vector was designed where the foreign sequence would replace the natural  $\beta\text{B}$ - $\beta\text{C}$  loop in VP23 rather than be added to it. In the nucleotide sequence of the region of the CPMV genome encoding VP23 a single silent base change (U to C) at position 2740 creates a unique

Aat11 site at amino acid valine 27. The change in the sequence of M RNA is shown in Figure 8. The creation of the Aat11 site enables the nucleotide sequence encoding the six amino acids from the native  $\beta$ B- $\beta$ C loop in CPMV to be removed by digestion with Nhe1 and Aat11. The sequence can then be replaced by any sequence with Nhe1- and Aat11-compatible ends.

Two different sequences were designed to be substituted for the sequence between the Nhe1 and Aat11 sites of the mutated M RNA sequence. The first sequence to be substituted into VP23 consisted of oligonucleotides encoding residues 735-752 from the transmembrane glycoprotein gp41 from human immunodeficiency virus (HIV-1). This sequence was selected as a synthetic peptide for this region is recognised in enzyme-linked immunosorbent assays (ELISA) by antisera from seropositive AIDS patients and is capable of inducing antibodies which neutralise a range of HIV-1 isolates (Kennedy *et al.*, 1986; Chanh *et al.*, 1986; Dagleish *et al.*, 1988). The second sequence consists of the nucleotide sequence encoding residues 85-99 from VP1 of human rhinovirus 14 (HRV14). In both cases, the oligonucleotides were designed to contain restriction enzyme sites to facilitate screening. The sequences of the oligonucleotides and the effect of the substitutions on the amino acid sequence of VP23 are shown in Figures 9 and 10. The steps in the construction of pMT7-HIV and pMT7-HRV are given below and are shown diagrammatically in Figure 11.

STEP 1. M13-JR-1 (see Figure 6) was propagated in *E. coli* strain CJ236 and dU-containing single-stranded DNA isolated as described by Kunkel (1985). The T to C mutation at position 2740 of the M RNA sequence was made by oligonucleotide-directed mutagenesis of dU-containing single-stranded M13-JR1 DNA using the primer CTG-CTG-TGA-CGT-CTG-AAA-A as described by Kunkel (1985). This resulted in the construction of clone M13-JRAat11. The mutation was confirmed by dideoxy sequence analysis of single-stranded DNA (Biggin *et al.*, 1983) and by restriction enzyme digestion of the double-stranded replication form DNA.

STEP 2. The replicative form DNA of M13-JRAat11 was isolated and digested



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with Nhe1 and Aat11 and treated with calf intestinal phosphatase. The pairs of oligonucleotides shown in Figures 9 and 10 were phosphorylated with ATP using polynucleotide kinase, annealed together by boiling and slow cooling and ligated into Nhe1/Aat11-digested M13-JRAat11. Recombinant M13 clones harbouring the inserted sequences were identified by sequence analysis of the single-stranded bacteriophage DNA exactly as described previously for pFMDV. Two clones, M13-HIV and M13-HRV containing the required sequences were identified and the double-stranded replicative form DNA was isolated shown to give the expected pattern of fragments on restriction enzyme digestion.

STEP 3. Replicative form DNA from M13-HIV and M13-HRV was digested with Sst1 and the 1.2kb M RNA-specific fragment recovered by electro-elution after electrophoresis of the digest on an agarose gel. The 1.2kb fragments were ligated into the large Sst1 fragment from pMT7-601 as previously described for production of pMT7-FMDV-I. The ligation mixture was used to transform E. coli strain JM83 and carbenicillin-resistant colonies selected. Two clones, designated pMT7-HIV and pMT7-HRV, were shown to contain the desired structure by restriction enzyme mapping and nucleotide sequence analysis.

For transcription, pMT7-HIV and pMT7-HRV, were linearised by digestion with EcoR1. Transcription using T7 RNA polymerase was carried out exactly as described for pMT7-601 and pBT7-123. The resulting transcripts were identical in size to natural virion RNA.

**Demonstration of the ability of pMT7-FMDV-I and pMT7-HIV transcripts to replicate in cowpea protoplasts**

10µg samples of the in vitro transcripts from either pMT7-601, pMT7-FMDV-I or pMT7-HIV were mixed with 15µg samples of transcripts from pBT7-123 and the mixtures used to electroporate 10<sup>6</sup> cowpea mesophyll protoplasts. Samples were either taken immediately (0 hour) or after incubation of the protoplasts for 72 hour in the light at 25°C. Nucleic acids were extracted from one quarter of each

sample and electrophoresed on a 1% formaldehyde-containing agarose gel as previously described. The nucleic acids were blotted on to Hybond N, cross-linked to the membrane by u.v. irradiation and probed for CPMV M RNA-specific sequences as previously described. In each case a strong hybridisation signal corresponding in position to M RNA could be detected in the 72 hour but not the 0 hour incubation samples, demonstrating that the transcripts from all four constructs can multiply in cowpea protoplasts.

The remaining three quarters of each protoplast sample was lysed as previously described and applied to electron microscope grids coated with anti-CPMV serum. The grids were then examined using a JEOL 1200 electron microscope. Large numbers of particles could be seen in the 72 hour samples of protoplasts electroporated with pMT7-601, pMT7-FMDV-I and pMT7-HIV transcripts. These results show that the modified coat proteins encoded by pMT7-FMDV-I and pMT7-HIV can assemble into virions.

**Ability of pMT7-FMDV-I and pMT7-HIV transcripts to replicate in whole cowpea plants**

To demonstrate the ability of transcripts from pMT7-FMDV-I and pMT7-HIV to replicate in whole cowpea plants in the presence of transcripts derived from pBT7-123, transcripts capped with GpppG were prepared as previously described. 6 groups, each consisting of 5, 10 day old, cowpeas, were inoculated with the transcripts using the method previously described. In each case, the amount of transcript refers to the amount applied to an individual leaf.

- |          |  |
|----------|--|
| Group 1. | Mock-inoculated with 50mM Tris-phosphate, pH 8.0               |
| Group 2. | Inoculated with 1.5µg of natural CPMV virion RNA               |
| Group 3. | Inoculated with 5µg each of pMT7-601 + pBT7-123 transcripts    |
| Group 4. | Inoculated with 5µg each of pMT7-FMDV-I + pBT7-123 transcripts |
| Group 5. | Inoculated with 5µg each of pMT7-HIV + pBT7-123 transcripts    |

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Symptoms were scored on a daily basis and samples of leaf tissue from each plant were taken 11 days post-inoculation for "dot blot" analysis which was carried out as described previously. The rest of the leaf tissue from all the plants in groups 4 and 5 was harvested and frozen for future use.

### Results

None of the plants in group 1 (mock-inoculated) developed any symptoms up to 11 days post infection (P.I.) and no CPMV-specific nucleic acids could be detected in the leaf tissue by "dot blot" analysis. This shows that no accidental infection of the cowpea plants with CPMV had occurred during the experiment. All plants in groups 2 and 3 (inoculated with either virion RNA or a mixture of pMT7-601 and pBT7-123 transcripts) showed strong symptoms on both the inoculated and systemic leaves by 7 days P.I. "Dot blot" analysis of leaf tissue showed the presence of large amounts of virus-specific RNA in both the inoculated and systemic leaves of all plants. This confirms that the plants used in the experiment were fully susceptible to infection with CPMV using either virion RNA or a mixture of wild-type transcripts.

By 11 days P.I. the inoculated leaves of all the plants in group 4 (inoculated with pMT7-FMDV-1 transcripts) developed a mottled appearance distinct from that normally associated with a wild-type virus infection. This result shows that the transcripts from pMT7-FMDV-1 can multiply and spread from cell-to-cell in whole cowpea plants.

4 out of 5 of the plants in group 5 (inoculated with pMT7-HIV transcripts) developed symptoms on their systemic leaves by 11 days P.I. "Dot blot analysis showed that plants showing symptoms had substantial quantities of virus-specific sequences in both the inoculated and systemic leaves. This result shows that transcripts from pMT7-HIV can multiply and spread within whole cowpea plants.

Further investigation of the results obtained with pMT7-FMDV-I and pMT7-HIV

pMT7-FMDV-I: To demonstrate that modified viral capsid proteins were synthesised in the inoculated leaves of the group 4 plants, samples of the frozen leaf tissue were finely ground and extracted with 1X Laemmli sample buffer. The extracts were electrophoresed on 15% polyacrylamide-SDS gels and the proteins transferred to nitrocellulose membranes using a Biorad semi-dry transfer cell. The membranes were probed either with serum raised against whole CPMV virus particles or with a serum raised against the synthetic oligopeptide, VPNLRGDLQVLAQKVARTLP(CG), corresponding to residues 141-160 of VP1 of FMDV strain O<sub>1</sub>. This sequence corresponds to the epitope which was inserted into VP23 in pMT7-FMDV. Both antisera were raised in rabbits. Western blot analysis was carried out using alkaline phosphatase-conjugated goat anti-rabbit IgG as the second antibody. The protein extracts of all five group 4 plants were found to react with the anti-CPMV serum indicating that the virus coat proteins were synthesised in the inoculated leaves of the group 4 plants. When similar blots were probed with the anti-FMDV oligopeptide serum, a single band lit up in the extracts from each of the group 4 plants (Figure 11). This band migrated with an apparent molecular weight of 24kDa, which is exactly the size expected for VP23 carrying the FMDV loop. No product of similar size could be seen when extracts from mock-inoculated or wild-type CPMV-inoculated leaves were analysed (Figure 11). Likewise, purified wild-type CPMV coat proteins did react with the FMDV-specific antiserum. Furthermore, pre-treatment of the anti-FMDV serum with the peptide which was used to raise it, abolished the reaction with the extracts from the group 4 plants demonstrating the specificity of the immunological reaction. These results demonstrate that the inoculated leaves of the group 4 plants contained CPMV coat proteins harbouring the FMDV loop.

pMT7-HIV: As discussed above, the "dot blot" analysis of both the inoculated and systemic leaves from the Group 5 plants indicated that transcripts from pMT7-HIV can multiply and spread in whole plants. The levels of signal obtained and the fact that the infection went systemic show that the progeny RNA is encapsidated. To prove that the HIV-specific insert was retained in the progeny

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RNA, "dot blots" of extracts from the group 5 plants were probed with a HIV-insert specific probe. This was made by "oligo-labelling" the positive sense oligonucleotide used in the construction of pMT7-HIV (see Figure 9). The results obtained showed the presence of the HIV sequence in extracts of the inoculated leaves of the four plants which showed symptoms.

#### **EXTENSION OF RESULTS WITH CPMV HARBOURING THE FMDV EPI TOPE**

To extend the previous findings obtained with transcripts derived from pMT7-FMDV-I, five groups of five cowpea plants were inoculated with capped transcripts, prepared as previously described, as follows:

- Group 1: Mock-inoculated with 50mM Tris-phosphate, pH 8.0
- Group 2: Inoculated with 0.5µg of natural CPMV virion RNA
- Group 3: Inoculated with 5µg GpppG capped pMT7-601 + pBT7-123 transcripts
- Group 4: Inoculated with 5µg GpppG capped pMT7-FMDV-I + pBT7-123 transcripts

Symptoms were scored on a daily basis. 13 days post-inoculation triplicate leaf disk samples were taken from one inoculated and one trifoliate leaf of each plant. The samples were treated as follows:

Sample 1 (Crude homogenate): Homogenised in 0.4mls 10mM sodium phosphate buffer, pH7.0, centrifuged and the supernatant recovered.

Sample 2 (RNA extract): Frozen in liquid nitrogen, finely ground and the nucleic acids extracted with phenol/chloroform. After ethanol precipitation, the nucleic acids were finally resuspended in 0.1mls of water.

Sample 3 (Protein extract): Frozen in liquid nitrogen, finely ground and the powder dissolved in 0.1ml 1X Laemmli sample buffer.

"Dot blots" were prepared from 5µl aliquots of samples 1 and 2 and were probed with either a probe specific for nucleotides 482-2211 of CPMV M RNA (CPMV-specific probe), prepared as described previously, or with a probe specific for FMDV-specific insert. The latter was prepared by "oligo-labelling" the positive sense oligonucleotide shown in Figure 5. Western blots were prepared from aliquots of sample 3 and probed for FMDV-specific epitopes as described previously. ISEM was carried out on aliquots of sample 1.

### Results

No symptoms developed on any of the group 1 (mock inoculated) plants. Dot blots of crude homogenates or RNA extracts revealed no CPMV-specific or FMDV-specific sequences were present in extracts from either the inoculated or trifoliate leaves. ISEM of the crude homogenates using electron microscopy grids coated with anti-CPMV serum showed no virus particles were present. Western blot analysis of the protein extracts using the FMDV-specific serum showed an absence of any FMDV epitopes. These results provide the negative control for the rest of the experiment.

Symptoms developed on both the inoculated and trifoliate leaves of all group 2 (virion RNA-inoculated) and group 3 (pMT7-601 + pBT7-123) -inoculated plants by 7 days PI. By 11 days PI the lesions on the primary leaves had expanded to a diameter of 2-3 mm. Dot blots of both the crude homogenates and RNA extracts revealed the presence of CPMV-specific but not FMDV-specific sequences. ISEM using grids coated with anti-CPMV serum revealed the presence of copious numbers of CPMV particles in crude homogenates from both the inoculated and trifoliate leaves. Western blot analysis of the protein extracts showed an absence of any FMDV epitopes.

Small lesions (approximately 1mm in diameter) developed on the inoculated leaves of the group 4 (pMT7-FMDV-I + pBT7-123)-inoculated plants by 11 days PI. Dot blots of the RNA extracted from the leaves (sample 2) showed the presence of both CPMV- and FMDV-specific sequences in the inoculated of 3 out

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of the 5 group 4 plants. ISEM using grids coated with anti-CPMV serum revealed the presence of CPMV-like virus particles in the crude homogenates from the inoculated leaves of 4 out of the 5 group 4 plants. Western blotting of the protein extracts (sample 3) revealed the presence of the FMDV epitope on the small coat protein in extracts from all group 4 plants. These results confirm that transcripts from pMT7-FMDV-I can multiply in whole cowpea plants and show that virus particles are produced in such plants.

#### **PREPARATION OF VIRUS FROM TRANSCRIPT-INOCULATED PLANTS**

To isolate virus particles from pMT7-FMDV-I-infected leaf tissue the following method was developed:

22 grams of primary cowpea leaves which had been inoculated with 5µg each of pBT7-123 and pMT7-FMDV transcripts were harvested 16 days post-inoculation. The leaves were homogenised in 2 volumes (approximately 50mls) of 0.1M sodium phosphate, pH 7.0 at 4°C. The sap was filtered through two layers of muslin, centrifuged at 15,000g for 15 minutes and the supernatant retained. The pellet was re-extracted with a few mls of 0.1M sodium phosphate buffer pH 7.0, re-centrifuged. The supernatants were combined and centrifuged in a Beckman type 30 rotor at 27,000 rpm for 4 hours at 4°C. The resulting pellet was resuspended overnight at 4°C in 3.5mls of 0.1M sodium phosphate, pH 7.0 and subsequently centrifuged in an Eppendorf centrifuge for 10 minutes. The supernatant was taken and made up to 4 mls with 0.1M sodium phosphate pH 7.0 and 1ml of a solution containing 1M NaCl, 20% PEG 6000 was added and the mixture incubated for 2 hours at room temperature. The resulting precipitate was collected by centrifugation in an Eppendorf centrifuge for 10 minutes, resuspended in 0.25mls of 10mM sodium phosphate, pH 7.0 and the solution clarified by re-centrifugation in an Eppendorf centrifuge for 10 minutes. The supernatant, which contains the virus particles, was then removed and stored at 4°C. It was estimated spectrophotometrically that the virus concentration in the final suspension was approximately 1.5 mg/ml. Western blot analysis of

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the virus using FMDV-specific antiserum revealed the presence of FMDV antigen associated with the small coat protein subunit of the chimaeric virus particles.

### **PASSAGING MODIFIED RNA**

In order to produce large quantities of chimaeric virus as efficiently as possible, RNA extracted from transcript-inoculated leaves was passaged in plants. 5µl samples of the RNA extract from a pMT7-FMDV-I-inoculated leaf were diluted to 50µl with Tris-phosphate pH 8.0 and were inoculated on to the primary leaves of a batch of 5 cowpea plants. All the plants developed symptoms typical of a CPMV infection and at 23 days PI the primary leaves from the plants were harvested. The leaves were homogenised in 0.1M sodium phosphate buffer and virus extracted as described above except that the initial high speed pelleting step was omitted. A total of 3.0 mgs of virus at a final concentration of 0.5mg/ml in 10mM sodium phosphate pH7.0 was isolated in this way. This preparation was finally concentrated in a Centriprep concentrator (Amicon) to a final concentration of 1.4 mg/ml and has been designated P1.

Samples of P1 were examined by electrophoresis on SDS gels and Coomassie Blue staining and shown to contain the expected pattern of coat proteins. Western blot analysis using anti-FMDV serum showed the small coat proteins contained the FMDV loop. RNA extracted from the virus particles was of the expected size for M and B RNA of CPMV. This demonstrates that chimaeric virus can be produced by passaging the RNA derived from transcript-inoculated leaves.

An experimental vaccine was prepared from virus preparation P1 by dispersion in sterile phosphate buffered saline (PBS) at a final concentration of 1 mg/ml. Guinea pigs were injected with 40 µg of the P1 vaccine on days 0 and 28. Preliminary results indicate that the animals produce antibodies against the FMDV loop, a response not seen when wild-type virus is injected.



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**CLAIMS**

1. Assembled particles of a plant virus containing a predetermined foreign peptide as part of the coat protein of the virus.
2. Virus particles according to claim 1, in which the foreign peptide is a biologically functional peptide, the biological application of which requires or is enhanced by presentation of the peptide in association with a larger molecule or particle.
3. Virus particles according to claim 1 or 2, in which the peptide is an antigen.
4. Virus particles according to claim 3, in which the antigen is a viral antigen.
5. Virus particles according to claim 4, in which the antigen is an animal (including human) viral antigen.
6. Virus particles according to any of claims 1 to 5, in which the foreign peptide is incorporated in an exposed surface of the coat protein of the plant virus.
7. Virus particles according to any of claims 1-6, wherein the plant virus is an RNA virus.
8. Virus particles according to any of claims 1 to 7, wherein the viral coat protein has a  $\beta$ -barrel structure.
9. Virus particles according to claim 8, wherein the foreign peptide is inserted in a loop connecting  $\beta$  sheets of the plant virus.

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10. Virus particles according to any of claims 1 to 9, in which the plant virus is a comovirus.
11. Virus particles according to claim 10, in which the comovirus is cowpea mosaic virus (CPMV).
12. Virus particles according to claim 9, 10, or 11, in which the foreign peptide is inserted in the  $\beta$ B- $\beta$ C loop of the plant virus.
13. Virus particles according to any of claims 9 to 12, in which the inserted foreign peptide is an addition to the existing loop.
14. Virus particles according to any of claims 9 to 12, in which the inserted foreign peptide is a replacement of part of the existing loop.
15. Virus particles according to any of claims 1 to 14, in which the foreign peptide is an animal virus antigen derived from Foot and Mouth disease virus (FMDV).
16. Virus particles according to any of claims 1 to 14, in which the foreign peptide is an animal virus antigen derived from human immune deficiency virus (HIV).
17. Virus particles according to any of claims 1 to 14, in which the foreign peptide is an animal virus antigen derived from a human rhinovirus (HRV).
18. A vaccine for protection against human or animal pathogens comprising assembled plant virus particles according to any of claims 3-17 as an immunogenic component thereof.
19. An animal virus vaccine comprising virus particles according to claim 5 as an immunogenic component thereof.

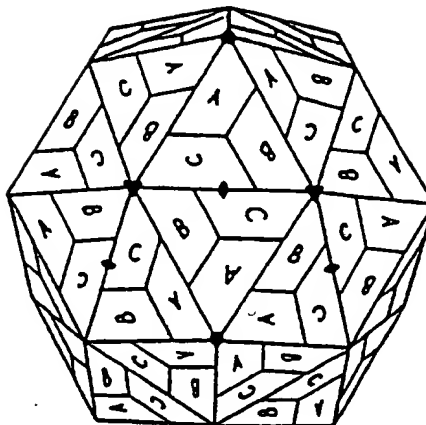
20. A method of producing plant virus particles according to any of claims 1 to 17, which comprises introducing a nucleotide sequence coding for a foreign peptide to modify the plant viral nucleic acid which codes for the coat protein, infecting plants, plant tissue, plant cells, or protoplasts with the modified viral nucleic acid, and harvesting assembled particles of the modified virus.
21. A method according to claim 20, in which the introduced nucleotide sequence is inserted in that part of the plant viral nucleic acid which codes for an exposed portion of the coat protein.
22. A method according to claim 20, applied to an RNA plant virus, which comprises introducing a DNA sequence coding for the foreign peptide into a cDNA corresponding to the RNA of the plant virus which codes for an exposed portion of its coat protein, producing from the thus modified cDNA an RNA transcript thereof, inoculating plants, plant tissue, plant cells, or protoplasts with the transcript, if necessary together with any other RNA required for multiplication and assembly of whole virus particles in the plant material, and harvesting assembled particles of the modified virus.
23. A method according to claim 22, in which the modified cDNA is produced by introducing the DNA encoding the foreign peptide into a DNA fragment excised from the plant viral cDNA, and recombining the modified fragment so as to reconstitute the plant viral cDNA in modified form.
24. A method according to claim 22 or 23, in which modified virus produced, or RNA extracted therefrom, is passaged in plants to produce further yields of modified virus.
25. A fragment of CPMV coat protein cDNA containing a DNA sequence encoding a foreign peptide at a site corresponding to an exposed surface of the coat protein.

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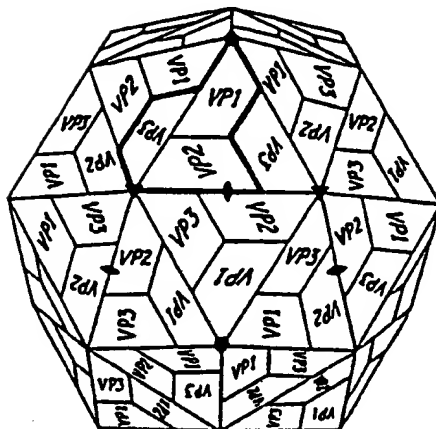
26. A fragment according to claim 25, being an Sst1 fragment.
27. A vector containing a fragment according to claim 25 or 26.
28. A vector comprising a full length cDNA of CPMV M RNA containing a DNA insert encoding the foreign peptide at a site corresponding to an exposed surface of the coat protein.
29. An RNA transcript of a fragment or vector according to any of claims 25 to 28.
30. A capped RNA transcript according to claim 29.
31. A method of protecting animals (including humans) against pathogens which comprises administering a vaccine according to claim 18 or 19.

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T=3



PICORNAVIRUS



COMOVIRUS

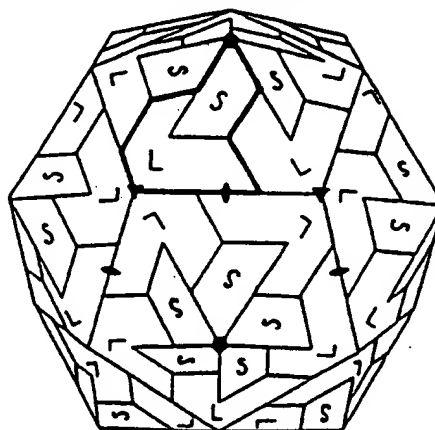
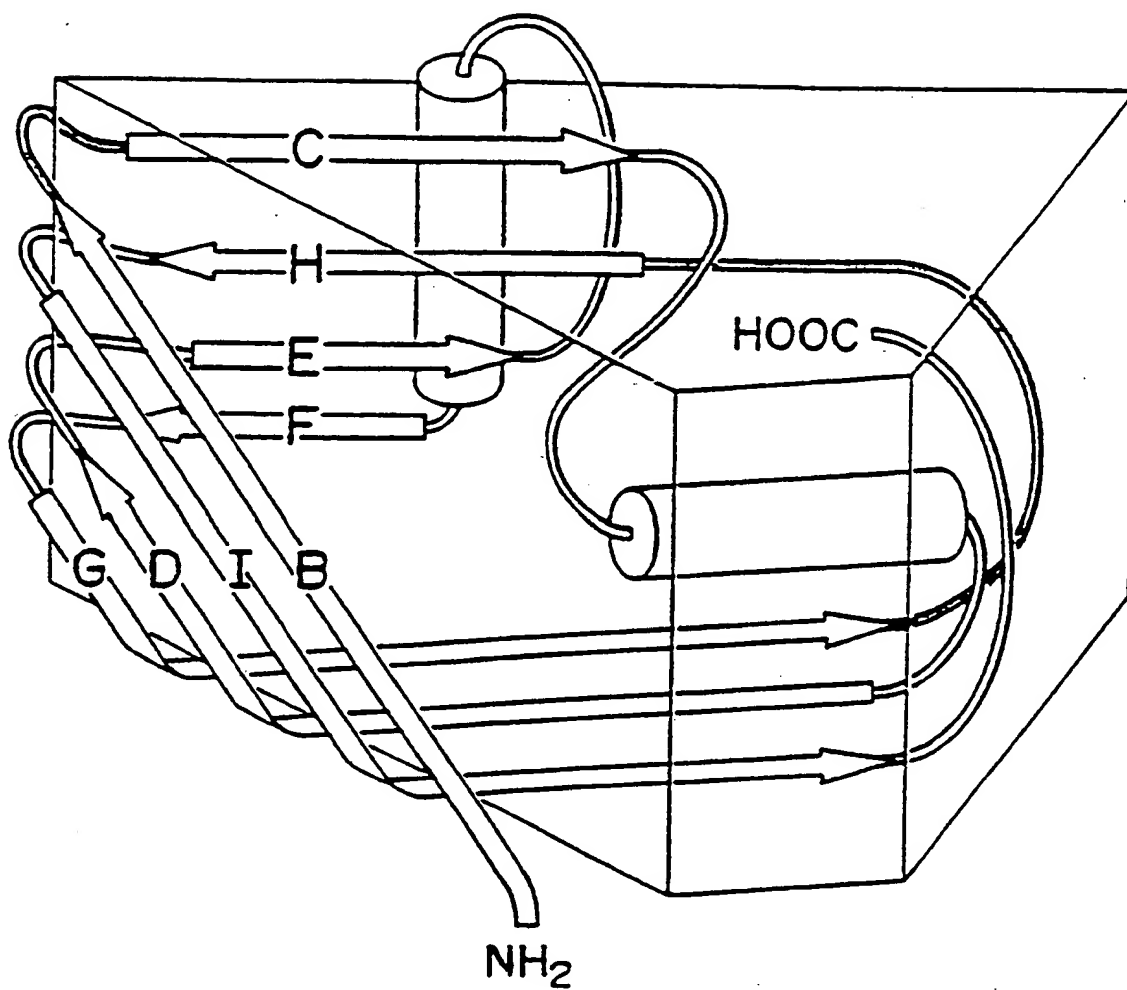


FIGURE 1

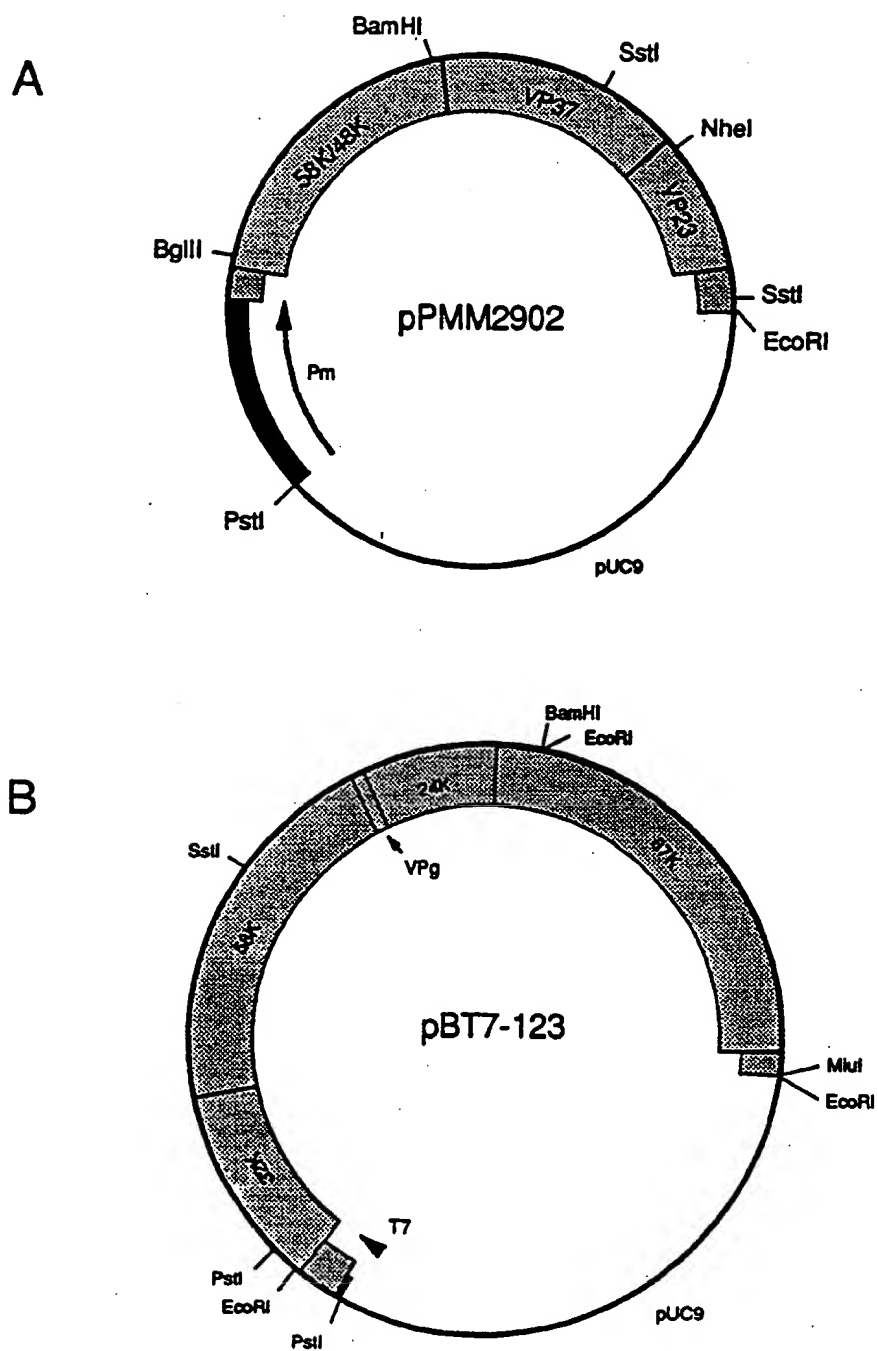
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FIGURE 2

SUBSTITUTE SHEET



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FIGURE 3

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1 5 10 15 20 25  
G P V C A E A S D V Y S P C M I A S T P P A P F S  
GGAOCTGTTTGTGCTGAAGCCTCAGATGTGTATAGCCCATGTATGATAGCTAGCACTOCTOCTGCTOCATTITCA  
2670 2700 Nhe1 2730

30 35 40  
D V T A V T F D L I N G K I T  
GACGTTACAGCAGTAACTTTTGACTTAATCAACGGCAAAATAACT  
2760

FIGURE 4

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(A)

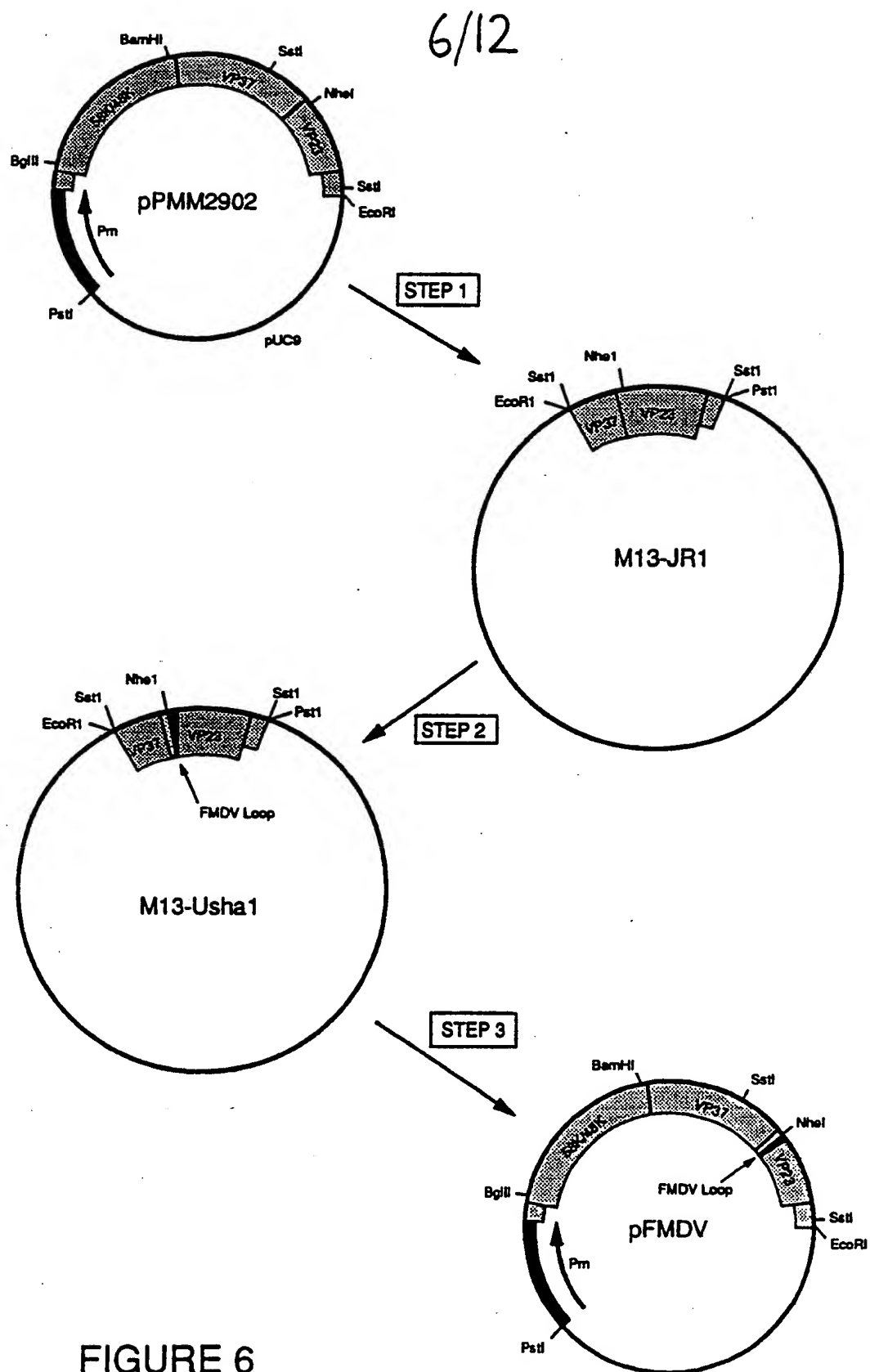
S T Y S R N A V P N L R G D L Q V L A Q K V A R T L P  
 CTAGCATTATAGTAGAAATGCTGTTCCTAATTGAGAGGAGATCTTCAAGTTTGGCTCAAAAGGTTGCTGGGACTCTTC  
 GTGAATATCATCTTTACGACAAGGATTAACTCTCTCTAGAAAGTTCAAAACOGAGTTTCCAAAGGAGGCTGAGAAGGATC  
BglII

(B)

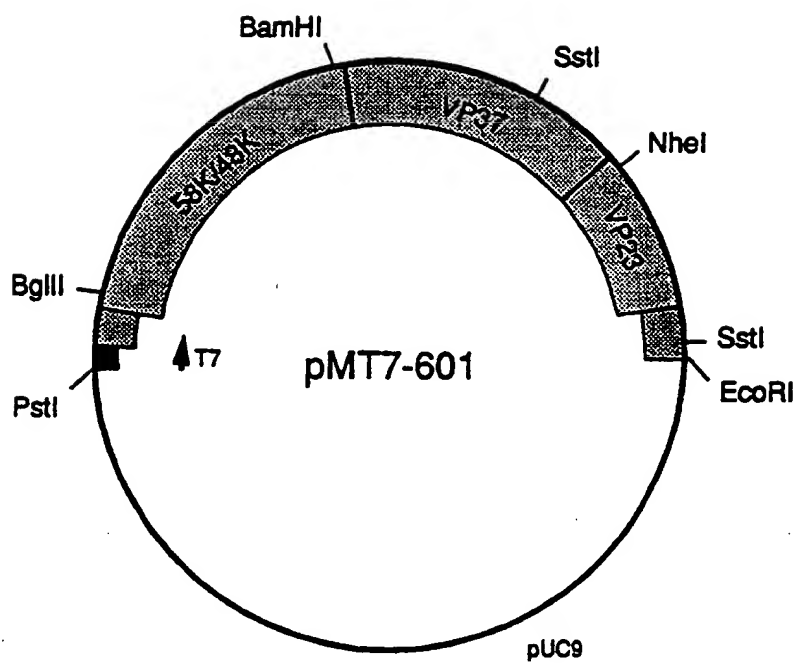
1 5 10 15 <-----  
 G P V C A E A S D V Y S P C M I A S T Y S R N A V P N  
 GGACCTGTGTTGTGCTGAAGGCTCAGATGTGTATAGCCCATGTATGATAGCTAGGCATTATAGTAGAAATGCTGTTCCTAAT  
 2670 2700 NheI

-----> 20 25  
 L R G D L Q V L A Q K V A R T L P S T P P A P F S  
 TTGAGAGGAGATCTTCAAGTTTGGCTCAAAAGGTTGCTGGGACTCTTCTAGCACTCTCTCTGCTCTCATTTCA  
BglII xNheI 2730

FIGURE 5

**FIGURE 6**

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FIGURE 7

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15 20 25 30 35  
P C M I A S T P P A P F S D V T A V T F D L I  
CCATGTATGATAGCTAGCACTCTCTCTGCTCCATTTTCAGACGTTACAGCAGTAACTTTTGACTTAATC  
2700 Nhe1 2730 \* 2760



Site-directed  
Mutagenesis

15 20 25 30 35  
P C M I A S T P P A P F S D V T A V T F D L I  
CCATGTATGATAGCTAGCACTCTCTCTGCTCCATTTTCAGACGTTACAGCAGTAACTTTTGACTTAATC  
2700 Nhe1 2730 Aat11 2760

FIGURE 8

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(A)

S T D R P E G I E E E G G E R D R D R S D  
 CTAGCACTGACCGGCGCTGAGGGCATOGAGGAAGAGGGGGGTGAGCGGATOGTGATOGTTGGGAOGT  
 GTGACTGGGGGACTCGCGTAGCTCTCTCTCGCGGCTAGCACTAGCAAGCC  
 Pvu1

(B)

1 5 10 15 <-----  
 G P V C A E A S D V Y S P C M I A S T D R P E G I E  
 GGACCTGTTTGTGCTGAAGCGTCAGATGTGTATAGCGCATGTATGATAGCTAGCACTGACCGGCGCTGAGGGCATOGAG  
 2670 2700 Nhe1

-----> 30 35  
 E E G G E R D R D R S D V T A V T F D L I  
 GAAGAGGGGGGTGAGCGGATOGTGATOGTTGGAGCTCAGCAGTAACCTTTGACTTAATC  
 Pvu1 Aat11 2760

FIGURE 9

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(A)

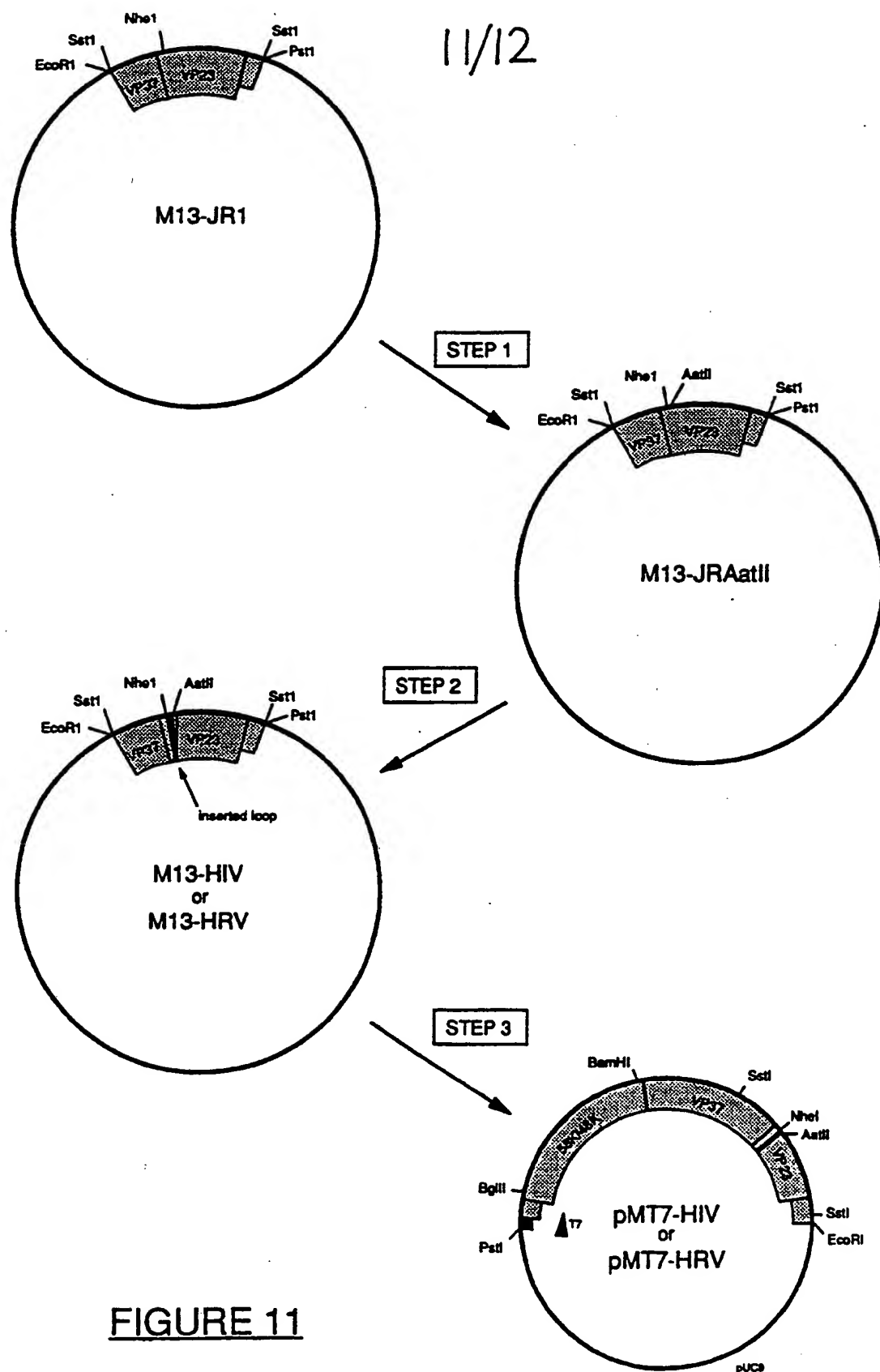
S T P A T G I D N H R E A K L D  
CTAGCACTCTGCTACTGGAATCGATAATCATAGAGAAGCTAAATTGGACGT  
GTGAGGAAGATGAOCCTTAGCTATTAGTATCTCTTGGATTTAACC  
Cla1

(B)

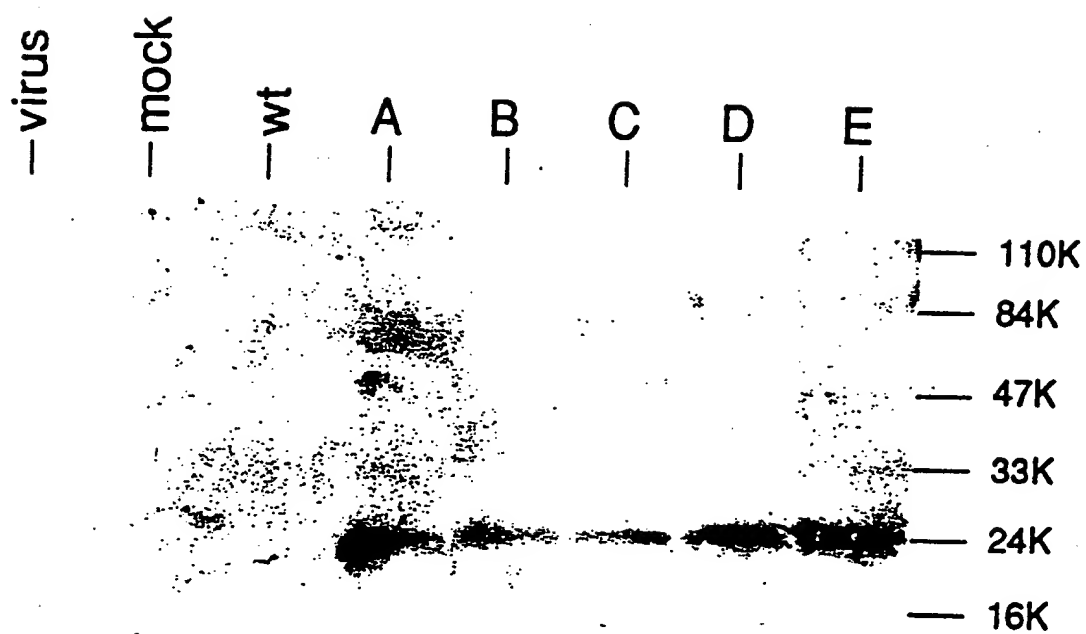
1 5 10 15 20 25 30 35  
G P V C A E A S D V Y S P C M I A S T P A T G I D N  
GGAACTGTTTGTGCTGAAGCCTCAGATGTGTATAGCCATGTATGATAGCTAGCACTCTCTGCTACTGGAATCGATAAT  
2670 2700 NheI ClaI  
H R E A K L D V T A V T F D L I  
CATAGAGAAGCTAAATTGGACGTCACAGCAGTAACITTTTGACITTAATC  
AatII 2760

FIGURE 10



**FIGURE 11**

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FIGURE 12

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 92/00589

| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup><br>According to International Patent Classification (IPC) or to both National Classification and IPC<br>IPC5: C 12 N 7/01, C 12 N 15/83, A 61 K 39/135, 39/21, 39/125<br>39/12   |   |                                     |   |  |                                     |                |   |                                 |                        |  |      |
|--|---|-------------------------------------|---|--|-------------------------------------|----------------|---|---------------------------------|------------------------|--|------|
| <b>II. FIELDS SEARCHED</b><br><div style="text-align: center; margin-top: 5px;">Minimum Documentation Searched<sup>7</sup></div> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%; border: none; padding: 5px;">Classification System</td> <td style="border: none; padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="border: none; padding: 5px; vertical-align: top;">IPC5</td> <td style="border: none; padding: 5px; vertical-align: top;">C 12 N; A 61 K</td> </tr> </table> <div style="text-align: center; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched<sup>8</sup></div>  |   |                                     | Classification System                                     | Classification Symbols   | IPC5                                | C 12 N; A 61 K |   |                                 |                        |  |      |
| Classification System  | Classification Symbols  |                                     |   |  |                                     |                |   |                                 |                        |  |      |
| IPC5   | C 12 N; A 61 K  |                                     |   |  |                                     |                |   |                                 |                        |  |      |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 5px;">Category</th> <th style="width: 70%; padding: 5px;">Citation of Document,<sup>11</sup> with indication, where appropriate, of the relevant passages<sup>12</sup></th> <th style="width: 20%; padding: 5px;">Relevant to Claim No.<sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">           NUCLEIC ACIDS RESEARCH, vol. 17, No. 7, 1989, R.J. Hayes et al: "Stability and expression of bacterial genes in replicating geminivirus vectors in plants", pp 2391-2403<br/><br/> <div style="text-align: center;">--</div> </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-3,6, 20-21</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">           EP, A2, 0278667 (LUBRIZOL GENETICS, INC.)<br/>           17 August 1988,<br/>           see the whole document<br/><br/> <div style="text-align: center;">--</div> <div style="text-align: center;">-----</div> </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-30</td> </tr> </tbody> </table>  |   |                                     | Category  | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup> | Relevant to Claim No. <sup>13</sup> | X              | NUCLEIC ACIDS RESEARCH, vol. 17, No. 7, 1989, R.J. Hayes et al: "Stability and expression of bacterial genes in replicating geminivirus vectors in plants", pp 2391-2403<br><br><div style="text-align: center;">--</div> | 1-3,6, 20-21                    | A                      | EP, A2, 0278667 (LUBRIZOL GENETICS, INC.)<br>17 August 1988,<br>see the whole document<br><br><div style="text-align: center;">--</div> <div style="text-align: center;">-----</div> | 1-30 |
| Category   | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>  | Relevant to Claim No. <sup>13</sup> |   |  |                                     |                |   |                                 |                        |  |      |
| X  | NUCLEIC ACIDS RESEARCH, vol. 17, No. 7, 1989, R.J. Hayes et al: "Stability and expression of bacterial genes in replicating geminivirus vectors in plants", pp 2391-2403<br><br><div style="text-align: center;">--</div> | 1-3,6, 20-21                        |   |  |                                     |                |   |                                 |                        |  |      |
| A  | EP, A2, 0278667 (LUBRIZOL GENETICS, INC.)<br>17 August 1988,<br>see the whole document<br><br><div style="text-align: center;">--</div> <div style="text-align: center;">-----</div>                                      | 1-30                                |   |  |                                     |                |   |                                 |                        |  |      |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><b>* Special categories of cited documents:<sup>10</sup></b></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div> |   |                                     |   |  |                                     |                |   |                                 |                        |  |      |
| <b>IV. CERTIFICATION</b> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border: none; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="border: none; padding: 5px;">17th June 1992</td> <td style="border: none; padding: 5px; text-align: center;">10 JUL 1992</td> </tr> <tr> <td style="border: none; padding: 5px;">International Searching Authority</td> <td style="border: none; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="border: none; padding: 5px; text-align: center;">EUROPEAN PATENT OFFICE</td> <td style="border: none; padding: 5px;">           Mme N. KUIPER  </td> </tr> </table>  |   |                                     | Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report  | 17th June 1992                      | 10 JUL 1992    | International Searching Authority   | Signature of Authorized Officer | EUROPEAN PATENT OFFICE | Mme N. KUIPER  |      |
| Date of the Actual Completion of the International Search  | Date of Mailing of this International Search Report   |                                     |   |  |                                     |                |   |                                 |                        |  |      |
| 17th June 1992   | 10 JUL 1992   |                                     |   |  |                                     |                |   |                                 |                        |  |      |
| International Searching Authority  | Signature of Authorized Officer   |                                     |   |  |                                     |                |   |                                 |                        |  |      |
| EUROPEAN PATENT OFFICE   | Mme N. KUIPER   |                                     |   |  |                                     |                |   |                                 |                        |  |      |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB92/00589

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 31  
because they relate to subject matter not required to be searched by this Authority, namely:  
See PCT Rule 39.1(iv)  
Methods for treatment of the human or animal body by  
surgery or therapy, as well as diagnostic methods
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such  
an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 92/00589

SA 57984

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on 29/05/92  
The European Patent office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|---------------------|----------------------------|---------------------|
| EP-A2- 0278667                            | 17/08/88            | AU-B- 606382               | 07/02/91            |
|   |                     | AU-D- 1138388              | 11/08/88            |
|   |                     | JP-A- 63301787             | 08/12/88            |

For more details about this annex : see Official Journal of the European patent Office, No. 12/82